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IL-4 and IL-10 Antagonize IL-12-Mediated Protection Against Acute Vaccinia Virus Infection with a Limited Role of IFN-γ and Nitric Oxide Synthetase 2[1]

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Resistance or susceptibility to most infectious diseases is strongly determined by the balance of type 1 vs type 2 cytokines produced during infection. However, for viruses, this scheme may be applicable only to infections with some cytopathic viruses, where IFN-γ is considered as mandatory for host defense with little if any participation of type 2 responses. We studied the role of signature Th1 (IL-12, IFN-γ) and Th2 (IL-4, IL-10) cytokines for immune responses against vaccinia virus (VV). IL-12$^{-/-}$ mice were far more susceptible than IFN-$\gamma^{-/-}$ mice, and primary CTL responses against VV were absent in IL-12$^{-/-}$ mice but remained intact in IFN-$\gamma^{-/-}$ mice. Both CD4$^+$ and CD8$^+$ T cells from IL-12$^{-/-}$ mice were unimpaired in IFN-γ production, although CD4$^+$ T cells showed elevated Th2 cytokine responses. Virus replication was impaired in IL-4$^{-/-}$ mice and, even more strikingly, in IL-10$^{-/-}$ mice, which both produced elevated levels of the proinflammatory cytokines IL-1α and IL-6. Thus, IL-4 produced by Th2 cells and IL-10 produced by Th2 cells and probably also by macrophages counteract efficient anti-viral host defense. Surprisingly, NO production, which is considered as a major type 1 effector pathway inhibited by type 2 cytokines, appears to play a limited role against VV, because NO sythetase 2-deficient mice did not show increased viral replication. Thus, our results identify a new role for IL-12 in defense beyond the induction of IFN-γ and show that IL-4 and IL-10 modulate host protective responses to VV. *The Journal of Immunology,* **2000, 164: 371–378.**

CD4$^+$ (Th) cells can be divided into at least two subsets of effector cells, termed Th1 and Th2, with contrasting cytokine profiles. Immune responses are often polarized, as cytokine secretion from one subset inhibits the development of the other (1). The distinctive cytokine profiles can be correlated well with functional differences of the two subsets, which has provided a concept for how the immune system combats such diverse pathogens as bacteria, protozoa, fungi, and helminthic parasites (2).

Anti-viral defense has been less dominated by the Th1-Th2 paradigm, because CD8$^+$ effector T cells are key mediators for clearance of noncytopathic viruses through cytolysis of infected cells and for clearance of cytopathic viruses through secretion of IFN-γ and TNF-α, which exert direct anti-viral activity (3, 4). Depending on the type of virus infection, CD4$^+$ T cells promote protection by providing help for B cells (3) and CTL responses (5, 6). In addition, a role of CD4$^+$ subpopulations (Th1 vs Th2) has been suggested, but is less well defined. For example, poliovirus-specific Th1 clones mediate protective immunity against a lethal poliovirus infection in a transgenic mouse model of poliomyelitis (7). CD4$^+$ T cells and IFN-γ can effectively clear mouse CMV infection in the absence of CD8$^+$ T cells (8–10). Similarly, influenza virus infection can be cleared in the absence of CD8$^+$ T cells (11), and Th1-specific anti-influenza clones protect against infection, whereas Th2 clones exacerbate pulmonary pathology (12). During measles virus infection, T cells show a biased Th2 response, which can be explained by the ability of the virus to suppress cell-mediated immunity and IL-12 production of dendritic cells and monocytes (13, 14). A prominent example is the case of HIV infection, where progression to AIDS may be associated with a Th1 to Th2 switch in a subset of patients (15, 16). In all of these examples, it has been suggested that Th1 responses promote viral clearance. IL-12 is the key cytokine for the induction of Th1 development (17), and IL-12 treatment has been shown to promote protective immunity to a variety of viruses including encephalomyocarditis virus (18), murine CMV (19), murine AIDS virus (20), lymphocytic choriomeningitis virus (LCMV)[3] (21), and hepatitis B virus (22). In contrast, experiments with IL-12$^{-/-}$ mice showed that endogenous IL-12 is not required for the control of mouse hepatitis virus (23) and LCMV infection (24).

Cell-mediated immune responses to microbial pathogens can be inhibited by Th2 cells. The best studied example is infection with *Leishmania major*, where IL-4 is responsible for fatal disease in susceptible BALB/c mice. Studies with viral infection systems have shown that overexpression of IL-4 using either recombinant vaccinia virus (VV) as a vector, IL-4 transgenic mice, or treatment with recombinant IL-4 was detrimental for the host during infection with VV (25), respiratory syncytial virus (26), and influenza virus (27). Further, transfer of influenza-specific Th2 clones delays virus clearance and exacerbates pulmonary pathology (12). Interestingly, several studies with IL-4$^{-/-}$ mice have failed to support a role of this cytokine in viral pathology (28–30).

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[3] Abbreviatios used in this paper: LCMV, lymphocytic choriomeningitis virus; VV, vaccinia virus; NOS2, NO synthetase 2; WR, Western Reservate strain of VV.

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IL-10, a cytokine secreted mainly by Th2 cells and macrophages, is a potent inhibitor of the inflammatory response and Th1 polarization during bacterial and parasitic diseases. Hardly anything is known about the role of IL-10 during virus infections, although the incorporation of the human IL-10 gene into the EBV genome (31) suggests that this cytokine may be important for the virus to evade host defense. However, the few studies that addressed this issue suggested that IL-10 does not interfere with virus clearance (32, 33).

Type 1 and type 2 cytokines have been shown to differentially regulate NO production. The release of NO by activated macrophages and neutrophils is one of the major defense weapons against many pathogens (34) including a number of viruses such as ectromelia virus (35), HSV (36), EBV (37), and coxsackie virus (38). However, the role of NO during VV infection appears intriguing. While NO can interrupt VV replication (39), and a recombinant VV encoding NO synthetase 2 (NOS2) is highly attenuated (40), pharmacologic inhibition of NO during VV infection does not alter the course of infection (41). Because pharmacologic inhibitors also affect NOS1 and NOS3, a detailed study on the role of NOS2 in combination with its major cytokine regulators remains to be done.

In this study, we have analyzed effector responses and virus clearance in C57BL/6 mice deficient for key type 1 cytokines (IL-12, IFN-γ) and type 2 cytokines (IL-4, IL-10), and for inducible NO synthase following infection with VV. VV is the representative member of the poxvirus family of cytoplasmic DNA viruses. VV induces cytolytic infections and is an expert in exploiting the cytokine network for immune evasion (42). Our results demonstrate that endogenous type 1 and type 2 cytokine responses cross-regulate immunity to acute VV infection with IL-12 and IL-10 as the dominant factors for resistance and susceptibility, respectively.

Materials and Methods

Mice

IL-4$^{-/-}$ mice (43), IL-10$^{-/-}$ mice (44) (kindly provided by W. Müller, Cologne, Germany), IFN-$\gamma^{-/-}$ mice (45) (kindly provided by Genentech, South San Francisco, CA), IL-12p35$^{-/-}$ mice (46) (kindly provided by J. Magram, Nutley, NJ), and NOS2$^{-/-}$ mice (47) (kindly provided by M. Modollel with the permission of J. Mudgett) were generated as described. Mutant mouse strains used for the experiments were back-crossed for six to eight generations to C57BL/6. Genotypes were determined by PCR amplification of DNA prepared from tail biopsies with specific oligonucleotide primers for IL-4 (forward, GTG AGC AGA TGA CAT GGG GC; reverse, CTT CAA GCA TGG AGT TTT CCC), IL-10 (forward, CAA AGC CAC AAG GCA GCC TTG; reverse, GAC AGT GCT AGA GCC CGG AGT), IFN-γ (forward, AGA AGT AAG TGG AAG GGC CCA GAA G; reverse, AGG GAA ACT GGG AGA GGA GAA ATA T), IL-12p35 (wild-type allele: forward, AGC TCC TCT CAG TGC CGG TC; reverse, GGT CTT CAG CAG GTT TCG GG; mutant allele: forward, GGC TCT GGA CTC ACC TGG AT; reverse, GCA TCG CAT TGT CTG AGT AGG), NOS2 (wild-type allele: forward, TCA CGC TTG GGT CTT GTT CAC; reverse, CAG GTC ACT TTG GTA GGA TTT; mutant allele: forward, GCA ATG TGA CAA AGC TCC TTC AG; reverse, GAA GAA CGA GAT CAG CAG CCT C). Mice were maintained in a facility free of specific pathogens at the Basel Institute for Immunology. C57BL/6 wild-type mice were purchased from IFFA-Credo (Saint Germain-sur-l'Abresle, France). For experiments, 8- to 12-wk-old female mice were used and kept in microisolator cages. At the time of experiments, 8-wk-old IL-10$^{-/-}$ mice showed no obvious signs of colitis.

Viruses and virus titration

VV strain Western Reservate (WR) and recombinant VV expressing either murine IL-4 (25), murine IFN-γ (48), or the LCMV glycoprotein (VV-G2) were grown at a low multiplicity of infection on BSC40 cells and plaqued on BSC40 cells. VV-IL-4 and VV-IFN-γ were kindly provided by A. Ramsay (Canberra, Australia). VV-G2 was used as a control recombinant VV, because G2 derived from LCMV is irrelevant for the anti-VV response. Mice were infected with 2×10^6 pfu VV i.p., unless stated otherwise, and sacrificed by CO_2. For determination of viral titers, lungs and ovaries were frozen at indicated days of harvest from mice and then thawed and homogenized in 2 ml MEM plus 2% FCS just before use in plaque assays. Tenfold dilutions were plaqued on monolayers of BSC40 cells in 24-well plates. Plates were stained after 48 h with crystal violet, and plaques were counted.

Cytotoxic T cell assay

Six days after infection, spleens were taken out and a single-cell suspension was made. Splenocytes were adjusted to 9×10^6 cells/ml. Target cells (MC57G H-2^b fibroblasts) were infected with VV-WR (multiplicity of infection, 3) for 3 h at 37°C. During the last 90 min of the infection, $^{51}CrNaCrO_4$ was added. Threefold dilutions of effector cells were incubated for 6 h at 37°C with 10^4 target cells in 200-μl cultures. The percentage specific ^{51}Cr release was calculated as: % specific lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%.

Separation and stimulation of cells

Spleens were removed at day 7 after infection, teased to single-cell suspensions, and CD4$^+$ and CD8$^+$ (mAb 53–6.72) T cells were purified by magnetic cell separation (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were incubated with CD4 mAb or CD8 mAb coupled to magnetic beads according to the manufacturer's instructions and sorted using MACS columns and the MidiMACS system. Aliquots of the unsorted and sorted cell fractions were analyzed by flow cytometry. CD4$^+$ and CD8$^+$ T cell populations were sorted to a purity of 80–90%. Cells were kept on ice until further use for in vitro cell culture. Purified cell populations and unsorted splenocytes from infected mice were plated (2×10^5/0.2 ml) in flat-bottom microtiter plates (Costar, Cambridge, MA) precoated with anti-CD3 mAb 145–2C11 (10 μg/ml) and cultured for 48 h. Cytokine levels in the supernatant were determined by sandwich ELISA for IL-4, IL-10, IFN-γ (PharMingen, San Diego, CA), IL-1α, and IL-6 (Genzyme, Cambridge, MA).

Histology

On day 6 after infection, lungs were isolated, fixed in 4% neutral buffered formalin, and paraffin embedded. Tissue sections (5 μm) were stained with hemotoxylin and eosin and analyzed microscopically. Photographs were taken with a Zeiss Axiophoto photomicroscope (Zeiss, Oberkochen, Germany).

Results

IL-12 is more important than IFN-γ for clearance of VV infection

To study the role of key type 1 cytokines on the susceptibility to infection with a cytopathic DNA virus, IL-12p35$^{-/-}$ mice, IFN-$\gamma^{-/-}$ mice, and C57BL/6 wild-type controls were infected with 10^6 pfu VV (WR), and virus titers were determined in ovaries, where VV replicates most extensively. VV clearance was markedly impaired in IL-12$^{-/-}$ mice, which showed a 20-fold and 100-fold increased viral load at days 3 and 6 after infection as compared with controls (Fig. 1). Virus titers were also highly elevated (10- to 20-fold) after infection with low-dose (10^4 pfu) VV (WR) (not shown). In contrast, clearance of VV (WR) was not severely affected in IFN-$\gamma^{-/-}$ mice throughout the course of infection (Fig. 1). Interestingly, after infection with a recombinant VV (VV-G2) (see Fig. 4), which is attenuated due to a targeted mutation of the thymidine kinase gene, virus titers in ovaries of IFN-$\gamma^{-/-}$ mice were ~10- to 50-fold increased compared with controls. Thus, our results demonstrate the IL-12 response to VV infection (25) is crucial for host defense, whereas IFN-γ appears neither required for virus clearance nor for the induction and reinforcement of this IL-12 response. However, dependent on the virulence of infection, IFN-γ can contribute to anti-viral immunity.

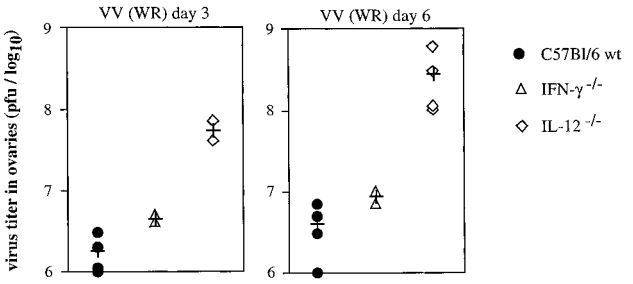

FIGURE 1. Impaired clearance of VV in IL-12$^{-/-}$ mice. Mice were infected i.p. with 10^6 pfu of VV (WR) and sacrificed on days 3 and 6 to collect ovaries for determination of virus titers. Shown are values of individual mice. Data are representative of three separate experiments.

Endogenous IL-4 and IL-10 inhibit clearance of VV infection

It has been well established that IL-4 and IL-10 can inhibit type 1 responses and prevent clearance of some pathogens (2). To assess the role of these cytokines during infection with VV, we measured virus titers in ovaries of IL-4$^{-/-}$ mice, IL-10$^{-/-}$ mice (both C57BL/6), and wild-type mice infected with varying doses (10^4, 10^6, and 10^8 pfu) of VV (WR) or with 10^6 pfu of recombinant VV-G2. A dose of 10^8 pfu was lethal for wild-type mice (3/3) and for IL-4$^{-/-}$ mice (2/3). In contrast, IL-10$^{-/-}$ mice showed a dramatically reduced virus titer ranging between 10^3- to 10^4-fold after infection with low, intermediate, and high doses of VV (WR) and VV-G2 at day 6 after infection (Fig. 2). Virus titers were reduced 10- to 20-fold as early as day 3 after infection. In IL-4$^{-/-}$ mice, virus titers in ovaries were reduced 80-fold after infection with 10^6 pfu VV (WR), whereas virus replication was not significantly different after infection with low doses (10^4 pfu) of VV (WR) or with 10^6 pfu of VV-G2 (Fig. 2). These results demonstrate that endogenous IL-4 and, in particular, IL-10 inhibit VV clearance. IL-4 production depends mainly on differentiated type 2 effector T cells producing a panel of type 2 cytokines including IL-10, suggesting that IL-4 may be important later in infection during the acquired response. In contrast, IL-10 can be produced by, and act as an autocrine inhibitor of, macrophages early after infection, which may explain the finding that IL-10 is more potent than IL-4 in suppression of host defense.

IFN-γ production does not require IL-12

Studies with mice deficient for IL-4 (43), IL-4Rα (49, 50), and STAT6 (51–53), the IL-4-activated signal transducer, have demonstrated an important role for IL-4 in the development of Th2 cells. Vice versa, mice deficient for IL-12 (46), IL-12Rβ1 (54), and STAT4 (55, 56), the IL-12-activated signal transducer, highlighted a critical role of IL-12 in the development of IFN-γ-secreting Th1 cells. To determine cytokine patterns in virus-infected mice, we purified splenic CD4$^+$ and CD8$^+$ cells from infected mice and measured IL-4, IL-10, and IFN-γ production after restimulation with anti-CD3. As shown in Fig. 3*A*, C57BL/6 mice mounted a strong type 1 response with IFN-γ produced by both CD4$^+$ and CD8$^+$ T cells. IL-4 was produced by CD4$^+$ T cells only, while IL-10 was secreted by CD4$^+$ and to a lower extent also from CD8$^+$ T cells. CD4$^+$ T cells in IL-12$^{-/-}$ mice showed augmented type 2 cytokine responses (e.g., IL-4 and IL-10), but also a slightly increased IFN-γ response. CD8$^+$ T cell cytokine production was unaltered in the absence of IL-12. CD4$^+$ T cells from IL-4$^{-/-}$ mice produced lower amounts of IL-10 but normal IFN-γ, indicating impaired Th2 without reciprocal increase in Th1 development. In contrast, Th1 development was markedly increased in IL-10-deficient mice. Next, we examined CD4$^+$ and CD8$^+$ T cells from bronchoalveolar lavage of infected mice. As determined by intracellular staining, the frequency of IFN-γ-producing CD4$^+$ T cells was unaltered in IL-4$^{-/-}$ mice (Fig. 3*B*), but was considerably enhanced in IL-10$^{-/-}$ mice and also slightly elevated in IL-12$^{-/-}$ mice (Fig. 3*B*), essentially in consistence with measurements in supernatants from restimulated splenic CD4$^+$ T cells. Thus, as might be predicted, we find enhanced Th1 development in the absence of IL-10 and impaired Th2 development in the absence of IL-4. Surprisingly, IFN-γ production was not significantly influenced by the absence of IL-12 or IL-4 after vaccinia infection.

To assess the production of proinflammatory cytokines, we measured IL-1α and IL-6 in supernatants after anti-CD3 stimulation of total splenocytes derived from groups of VV-infected mice. Both IL-4$^{-/-}$ and IL-10$^{-/-}$ mice showed markedly increased levels of both IL-1α and IL-6, (Fig. 3*C*), which demonstrates that both IL-4 and IL-10 are potent inhibitors of the proinflammatory cytokine response against VV.

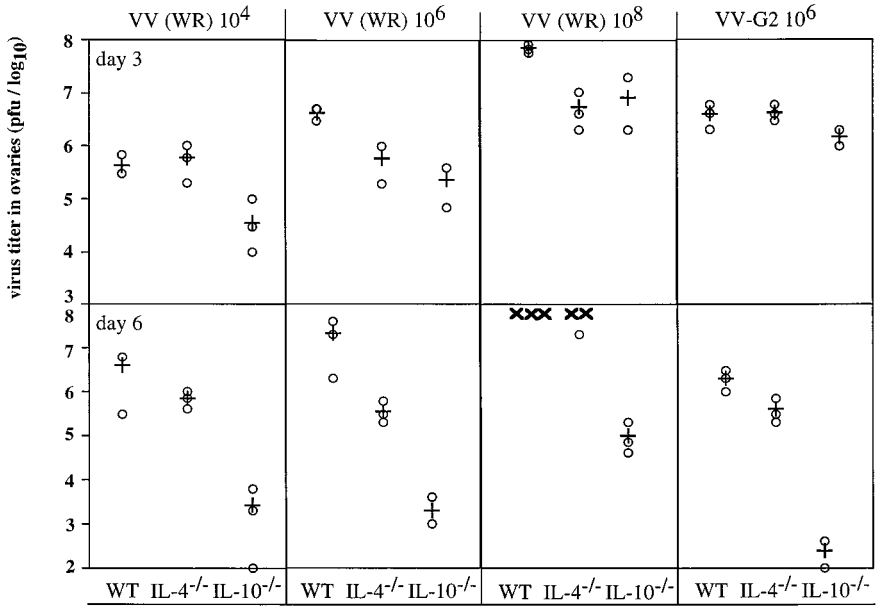

FIGURE 2. Enhanced clearance of VV in IL-4$^{-/-}$ and IL-10$^{-/-}$ mice. Mice were infected i.p. with doses indicated of VV (WR) and VV-G2 and sacrificed on days 3 and 6 to collect ovaries for determination of virus titers. Shown are values of individual mice. Data are representative of two separate experiments.

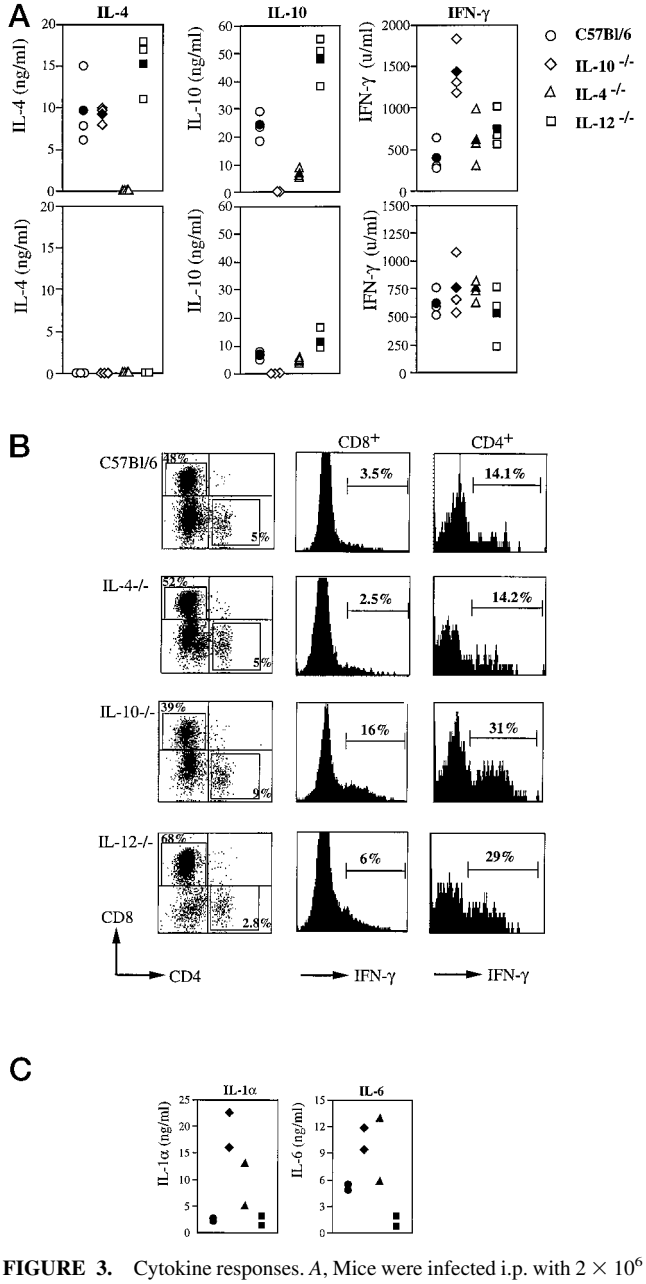

FIGURE 3. Cytokine responses. *A*, Mice were infected i.p. with 2×10^6 pfu of VV (WR) and sacrificed on day 7 to collect spleens and the bronchoalveolar lavage (BAL) for determination of cytokine production. Splenic $CD4^+$ T cells (*upper panel*) and $CD8^+$ T cells (*lower panel*) were purified by magnetic sorting and stimulated with immobilized anti-CD3 for 48 h before measurement of supernatants for IL-4, IL-10, and IFN-γ by ELISA. Shown are values of individual mice (open symbols) and the average of a group (filled symbols). *B*, BAL cells were stimulated with PMA and inonomycin for 4 h. During the last 2 h Brefeldin was added to retain cytokines in the cytoplasm. Cells were stained with APC-labeled anti-CD4, FITC-labeled anti-CD8, and after permeabilization using saponin, with PE-labeled anti-IFN-γ (or IL-4) before analysis by three-color flow cytometry. Shown is the percentage of $CD4^+$ and $CD8^+$ T cells producing IFN-γ. The frequency of IL-4-producing cells was below detection limit and is not shown. *C*, Unpurified splenocytes were stimulated as described above, and supernatants were measured for IL-1α and IL-6. Shown are average values (n = 3/group) from two different experiments.

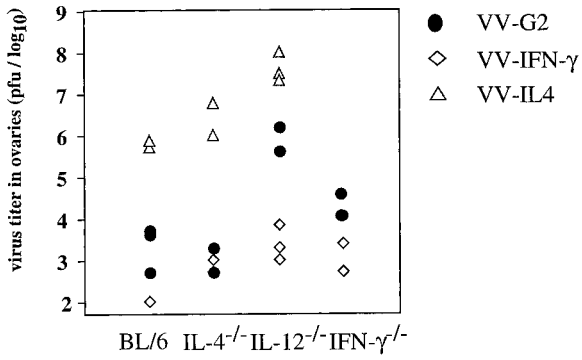

FIGURE 4. Mice were infected i.p. with 2×10^6 pfu of either VV-G2, VV-IL-4, or VV-IFN-γ and sacrificed on day 6 to collect ovaries for determination of virus titers. Shown are values of individual mice.

IL-4 expressed by recombinant VV affects virus clearance by inhibition of IL-12-dependent and IL-12-independent pathways

Ectopic expression of a cytokine exactly at the same time and place where the immune response against the virus is initiated allows one to study the potential effect of this cytokine on the generation of specific immunity and the consequences for virus control in vivo (57). To further understand the role of type 1 and type 2 responses, the effector mechanisms, and their interrelation during infection with VV, we infected wild-type and cytokine-deficient mice with 10^6 pfu recombinant VV expressing IL-4 (VV-IL-4), IFN-γ (VV-IFN-γ), or an irrelevant control protein (VV-G2) and determined virus clearance (Fig. 4) and virus-specific CTL responses (Fig. 5). In agreement with others, we found that virus-expressed IL-4 exacerbates VV infection in mice (25). Virus titers in ovaries of C57BL/6 mice were 100- to 1000-fold increased (Fig. 4) and persisted much longer (>11 days, not shown). Increased viral load (3 log) and delayed clearance was also observed in $IL\text{-}4^{-/-}$ mice, demonstrating that virus- but not host-encoded IL-4 inhibits virus clearance. Notably, virus titers in $IL\text{-}12^{-/-}$ mice were higher after VV-IL-4 compared with VV-G2 infection, suggesting that IL-4 inhibits other host defense pathways in addition to inhibition of IL-12. In contrast, the susceptibility of $IL\text{-}12^{-/-}$ and $IFN\text{-}\gamma^{-/-}$ mice to VV was almost entirely reverted by vector expressed IFN-γ (VV-IFN-γ).

CTL responses are abrogated in $IL\text{-}12^{-/-}$ mice but not in $IFN\text{-}\gamma^{-/-}$ mice

To evaluate the role of type 1 and type 2 cytokines on the activity of antiviral CTL responses, $IL\text{-}12^{-/-}$, $IFN\text{-}\gamma^{-/-}$, $IL\text{-}10^{-/-}$, and $IL\text{-}4^{-/-}$ mice were infected with VV-G2 and primary ex vivo CTL responses were measured at day 6, the peak time of anti-VV CTL (Fig. 5). In agreement with others, we found that IFN-γ is not required for the generation of cytolytic effector cells during virus infection (30, 58, 59). Indeed, vaccinia-specific CTL responses were slightly elevated in $IFN\text{-}\gamma^{-/-}$ mice, which may be due to enhanced proliferation of T cells in the absence of IFN-γ (45, 59). In contrast, CTL responses were virtually abrogated in infected $IL\text{-}12^{-/-}$ mice. $IL\text{-}4^{-/-}$ and $IL\text{-}10^{-/-}$ mice infected with recombinant VV-G2 showed little differences compared with wild-type mice. Basically the same results were obtained when the various cytokine-deficient mice were infected with VV (WR), with the exception of $IL4^{-/-}$ and $IL\text{-}10^{-/-}$ mice that showed slightly (~3-fold) enhanced CTL responses (data not shown). It has been suggested previously that IL-4 can suppress $CD8^+$ T cell cytotoxicity in vitro and in vivo. In agreement with these results, we found that CTL responses were abolished in immunocompetent mice infected with VV-expressing IL-4 (VV-IL-4). Surprisingly, VV-IL-4 failed to suppress CTL responses in $IL\text{-}4^{-/-}$ mice. In contrast, virus-expressed IFN-γ (VV-IFN-γ) reconstituted CTL responses to almost normal levels in $IL\text{-}12^{-/-}$ mice, which may suggest that

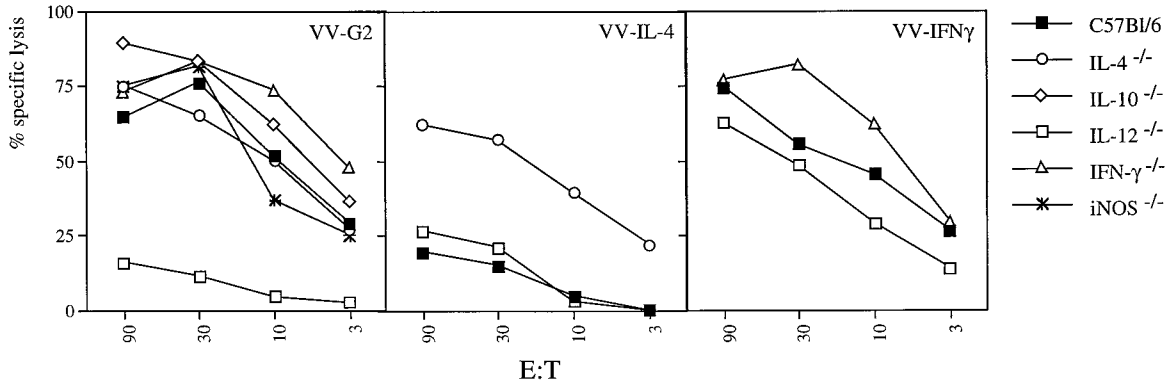

FIGURE 5. VV-specific primary CTL responses. Mice were infected i.p. with 2×10^6 pfu of either VV-G2, VV-IL-4, or VV-IFN-γ and sacrificed on day 6 to collect spleens for determination of primary CTL activity by ^{51}Cr release assay using MC57G fibroblasts infected with VV as target cells. Lysis of uninfected MC57G targets was <10%. Shown are values of the average of two mice. Data are representative of two separate experiments.

tissue damage and possibly $CD8^+$ T cell exhaustion due to increased viral replication is responsible for the lack of measurable CTL responses in IL-$12^{-/-}$ mice.

Severe morphological lung damage in IL-$12^{-/-}$ mice

We next assessed the overall pulmonary inflammatory process of mice infected with VV. Therefore, lung tissues from virus-infected mice were analyzed histologically. Lung pathology in C57BL/6 mice infected with VV-G2 was confined to a few foci of perivascular and peribronchial inflammation of lymphocytes, mononuclear, and some polymorphonuclear cells (Fig. 6). In contrast, infection of C57BL/6 and IL-$12^{-/-}$ mice with VV-IL-4 and VV-G2, respectively, profoundly altered the lung architecture, with extensive inflammation and edema, and with damage of alveoli.

NO is dispensable for clearance of VV

NO production has been shown to be beneficial as an anti-viral effector mechanism both in vitro and in vivo (34). Type 1 and type 2 cytokines are important regulators of NO production with antagonizing activities. IFN-γ and TNF-α are the main activators of NOS2, the enzyme that catalyzes NO production, whereas type 2 cytokines, i.e., IL-4, IL-10, and IL-13, are potent inhibitors. Thus, we hypothesized that the antagonizing effects of type 1 and type 2 cytokines on the control of VV infection may be mediated by regulation of NO production. To test the role of NO for clearance of acute VV infection, we studied NOS$2^{-/-}$ mice infected with VV (WR). Viral growth in the ovaries was comparable in NOS$2^{-/-}$ and C57BL/6 control mice at days 6 (Fig. 7), 8, and 10 (not shown), suggesting that NO production is not critical for control of VV infection.

Discussion

Our study demonstrates the first clear example where the balance of endogenous type 1 and type 2 cytokines produced in response to viral infection determines pathogen clearance and the severity of disease. IL-$12^{-/-}$ and IFN-$\gamma^{-/-}$ mice were more susceptible, whereas IL-$4^{-/-}$ and IL-$10^{-/-}$ mice were more resistant, to VV infection compared with wild-type controls. IFN-γ is considered as a critical player in anti-viral immune responses, mainly based on its direct anti-viral activity (4). However, in vivo studies have demonstrated that the role of IFN-γ in anti-viral immune responses varies with the type and conditions of infection. Mice with a disrupted gene for IFN-γR1 or IFN-γ, or mice treated with anti-IFN-γ Ab, develop increased susceptibility to VV (59), ectromelia virus (60), CMV (61), and mouse hepatitis virus (62, 63). In contrast, control of acute infection with vesicular stomatitis virus (59), pseudorabies virus (63), γ-herpesvirus (64), and influenza virus (58) is not dependent on IFN-γ. Although IFN-γ is thought to be crucial

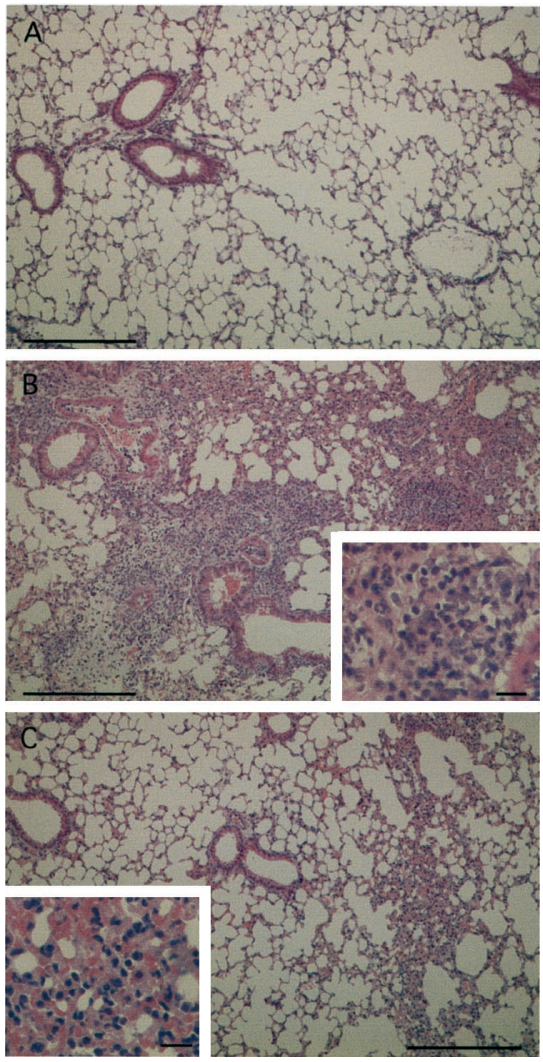

FIGURE 6. Characterization of lung pathology during VV infection. C57BL/6 (*A* and *C*) and IL-$12^{-/-}$ mice (*B*) were infected i.p. with 2×10^6 pfu of either VV-G2 (*A* and *B*) or VV-IL-4 (*C*) and sacrificed on day 6 to collect lungs for histological evaluation. Sections were stained with hemotoxylin and eosin.

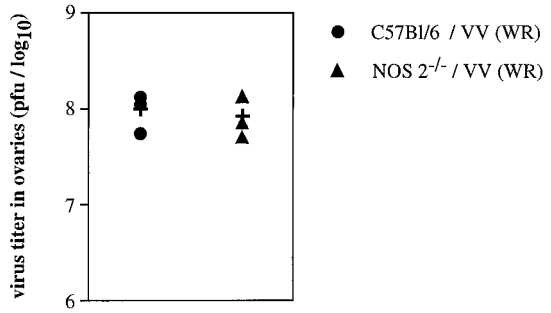

FIGURE 7. Unaltered VV clearance of NOS2$^{-/-}$ mice. Mice were infected i.p. with 2×10^6 pfu of VV (WR) and sacrificed on day 6 to collect ovaries for determination of virus titers. Shown are values of individual mice. Data are representative of two separate experiments.

for the control of VV (4), we found that endogenous IL-12 is superior to IFN-γ for virus clearance. IFN-$\gamma^{-/-}$ mice showed significantly increased susceptibility only to infection with VV strains of low virulence such as VV (Lancy) (65) or recombinant VV (i.e., VV-G2, with a mutation of the tk gene). Clearance of a strain with high virulence in mice was minimally affected by the absence of IFN-γ. In contrast, IL-12-deficient mice were much more susceptible, independent of the virulence of the VV strain. It has been shown that IL-12 treatment is beneficial for the host during infection with a variety of viruses, and that this effect is mediated primarily through induction of IFN-γ by NK cells and T cells (18, 20, 66, 67). Our results expand the host defense repertoire of IL-12 beyond the induction of IFN-γ, the most widely studied antimicrobial action of IL-12 (68). In addition to inducing IFN-γ, IL-12 has been shown to stimulate expression of TNF-α (69), which can act by itself in the absence of IFN-γ in the control of visceral leishmaniasis (70). An important role of TNF-α and the two receptors TNFR-1 (p55) and TNFR-2 (p75) in the control of VV replication has been demonstrated (57) .

CTL responses were abrogated in IL-12$^{-/-}$ mice but remained normal in IFN-$\gamma^{-/-}$ mice after VV infection. These findings are in concordance with those of others, who showed that in vitro CTL generation was augmented by the addition of IL-12 in the presence of neutralizing anti-IFN-γ Abs (71). Unimpaired CTL generation observed in IL-12$^{-/-}$ mice after immunization with allogeneic cells (46) or with LCMV (M. van den Broek, unpublished observations) probably reflects the inability of these stimuli to induce significant IL-12 levels in vivo (72) and an IL-12-independent pathway for the induction of CTL responses. High and low susceptibility to VV correlates with the absence and presence of CTL responses in IL-12$^{-/-}$ and IFN-$\gamma^{-/-}$ mice, respectively. This may argue that defective CTL responses allow uncontrolled VV replication in IL-12$^{-/-}$ mice. However, protection against acute and challenge infection with VV, vesicular stomatitis virus, and Semliki Forest virus (SFV) has been shown to be independent of both perforin- and fas-dependent pathways suggesting that CD8$^+$ T cell cytotoxicity plays a limited if any role after infection with cytopathic viruses (73).

Excess of IL-4 has been shown to be deleterious for the host in various virus models (25, 27, 29) In particular, overexpression of IL-4 at the site of infection using VV as a vector severely inhibits host defense (25). In agreement with these results, we found that C57BL/6 mice infected with recombinant VV-IL-4 developed lung pathology and virus titers similar to IL-12$^{-/-}$ mice infected with VV expressing an irrelevant control protein, suggesting that IL-12-mediated protection is inhibited by IL-4. However, the finding that VV-expressed IL-4 further increased viral spread in IL-12$^{-/-}$ mice argues that IL-4 has detrimental effects independent of IL-12 inhibition. Importantly, at physiological levels, IL-4 appears to play a more limited role as an inhibitor of VV clearance, which is dependent on the dose and virulence of the VV inoculate. Virus titers were significantly decreased in IL-4$^{-/-}$ mice infected with intermediate but not with low or high doses of VV (WR). Furthermore, infection with less virulent VV strains such as VV (Lancy) (28) or recombinant derivatives (i.e., VV-G2) showed no evident role for IL-4 in suppression of virus replication. Interestingly, we found that the absence of IL-10 promoted the host defense much stronger than the absence of IL-4. IL-10$^{-/-}$ mice showed markedly diminished virus titers independent of the dose and virulence of VV inoculate. Paradoxically, infection of mice with a recombinant VV-expressing murine IL-10 resulted in subtle in vivo differences (32). IL-10 is known as a potent inhibitor of proinflammatory cytokines and IL-12 production by both macrophages and dendritic cells in vitro (74, 75) and in vivo (76, 77). In agreement with this, we demonstrated that splenocytes of VV-infected IL-10$^{-/-}$ mice produced augmented levels of the proinflammatory cytokines IL-1 and IL-6. Splenocyte cultures of infected IL-4$^{-/-}$ mice also contained elevated levels of the two proinflammatory cytokines, which have been shown to be important for VV clearance (78). In vivo, IL-10 is probably a more effective inhibitor of VV clearance compared with IL-4, because it is secreted by activated macrophages and can act as an autocrine inhibitor immediately after infection, whereas IL-4 is produced mainly by Th2 cells that develop later in infection.

Based on the findings that NO production is induced by type 1 cytokines (e.g., IFN-γ) and inhibited by type 2 cytokines (IL-4, IL-10, IL-13, TGF-β) (34), we hypothesized that the difference in susceptibility of IL-12$^{-/-}$ and IFN-$\gamma^{-/-}$ vs IL-4$^{-/-}$ and IL-10$^{-/-}$ mice can be explained by altered regulation of NO. In fact, NO excess has been demonstrated to inhibit VV replication in vitro (39) and in vivo (40). Surprisingly, our results demonstrate that NOS2-deficient mice cleared VV as well as controls, which is in agreement with others showing that pharmacologic inhibition of NO during VV infection did not alter the course of infection (41). This argues that NO can exhibit potent anti-viral activity if expressed locally by recombinant VV, whereas it plays a limited if any role during the normal course of VV infection. Our results suggest that altered reactive nitrogen intermediates are not responsible for the susceptibility and the resistance of IL-12- and IL-10-deficient mice, respectively, to VV infection. The role of reactive oxygen intermediates in this scenario remains to be tested.

Immune responses to viruses are dominated by Th1 cells that provide help for anti-virus IgG2a responses and CTLs that kill infected cells. In general, virus-specific CD8$^+$ T cells produce the Th1-like cytokine pattern, i.e., IFN-γ, LT-α, and TNF-α, and these have been referred to as Tc1 cells. IL-12 has been shown to be a critical factor for the development of Th1 cells in response to bacteria and parasites (17, 79). Less is known about the role of IL-12 for Th1 and Tc1 development after viral infection. A variety of viruses have been shown to transiently increase the IL-12p40 gene or stimulate IL-12p70 production shortly after infection (80–82). However, IL-12$^{-/-}$ mice infected with mouse hepatitis virus (23) or LCMV (24) show unaltered IFN-γ and IL-4 responses in both CD4$^+$ and CD8$^+$ T cells. Our data demonstrate that IL-12 is dispensable for the development of both IFN-γ-producing CD4$^+$ and CD8$^+$ T cells after VV infection and that these cells also migrate to sites of virus replication. In contrast, in the absence of IL-12, we did find elevated Th2 cytokine production (i.e., IL-4 and IL-10) after VV infection. Previously it has been suggested that the presence of IL-4 can induce secretion of type 2 cytokines by CD8$^+$ T cells, which remain cytolytic or switch to noncytolytic Th cells

dependent on the experimental system (83, 84). Our present results suggest that $CD8^+$ Tc2 differentiation after viral infection is limited even when conditions appear favorable, i.e., the absence of IL-12 and elevated IL-4 secretion by $CD4^+$ T cells. Moreover, our results confirm that effector $CD8^+$ T cell cytolytic function appears not to be markedly affected by endogenous IL-4 levels as has been shown for VV, LCMV, and Sendai virus infection (28, 30), although the potential of IL-4, when present in excess, to inhibit CTL responses was evident after infection with recombinant VV-expressing IL-4 (25) or in IL-4 transgenic mice infected with respiratory syncytial virus (29). Inhibition of CTL by endogenous IL-4 may be more important during memory responses, as demonstrated for respiratory syncytial virus (85) .

Together our studies exemplify cytokine-mediated control of a cytopathic virus. IL-12 plays a dominant role in protective immunity to acute VV. IL-12 activity goes beyond the induction IFN-γ and includes control of CTL responses and probably innate immunity, such as the production of inflammatory cytokines (e.g.,TNF-α) and chemokines. These results warrant further studies with other cytolytic viruses such as influenza, which can be effectively controlled in the absence of IFN-γ (58). Endogenous IL-4 and, in particular, IL-10 responses interfere with immunity to VV, which can be explained by inhibition of the monokines IL-1, IL-6, and IL-12. Excessive amounts of IL-4 expressed by recombinant VV shows a more dramatic role than its absence during infection. Overexpression of IFN-γ by recombinant VV at the site of infection cures susceptible IL-$12^{-/-}$ mice (Fig. 4) and nude mice (4), demonstrating the potent direct anti-viral activity of IFN-γ. However, endogenous IFN-γ production is not crucial for control of VV replication. Interestingly, studies with recombinant VV vectors expressing IL-2 (86), TNF-α (87), CD40L (88), and NOS2 (40) have all suggested a critical role for these factors in resistance to VV. However, mice deficient for these genes cleared VV normally (28, 57, 89) (Fig. 7). In contrast, the role of endogenous IL-10 as an inhibitor of virus clearance was undiscovered in studies with recombinant VV-expressing IL-10. These results are important for understanding the role of type 1 and type 2 cytokines during infection with cytopathic viruses and for cytokine viral vector-based gene therapy and vaccination approaches.

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